Iron–sulphur cluster assembly in plants: distinct NFU proteins in mitochondria and plastids from *Arabidopsis thaliana*

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Recent results are in favour of a role for NFU-like proteins in Fe–S cluster biogenesis. These polypeptides share a conserved CXXC motif in their NFU domain. In the present study, we have characterized *Arabidopsis thaliana NFU1–5* genes. AtNFU proteins are separated into two classes. NFU4 and NFU5 are part of the mitochondrial type, presenting a structural organization similar to *Saccharomyces cerevisiae* Nfu1p. These proteins complement a *Δisu1 Δnfu1* yeast mutant and NFU4 mitochondrial localization was confirmed by green fluorescent protein fusion analysis. AtNFU1–3 represent a new class of NFU proteins, unique to plants. These polypeptides are made of two NFU domains, the second having lost its CXXC motif. AtNFU1–3 proteins are more related to *Synechocystis* PCC6803 NFU-like proteins and are localized to plastids when fused with the green fluorescent protein. NFU2 and/or NFU3 were detected in leaf chloroplasts by immunoblotting. NFU1 and NFU2 are functional NFU capable of restoring the growth of a *Δisu1 Δnfu1* yeast mutant, when addressed to yeast mitochondria. Furthermore, NFU2 recombinant protein is capable of binding a labile 2Fe–2S cluster *in vitro*. These results demonstrate the presence of distinct NFU proteins in Arabidopsis mitochondria and plastids. Such results suggest the existence of two different Fe–S assembly machineries in plant cells.

Key words: chloroplast, Fe–S, thale cress.

INTRODUCTION

Fe–S cluster assembly requires a set of proteins for the formation and transfer of this prosthetic group to an apoprotein [1]. In nitrogen-fixing bacteria, two polypeptides encoded by the Nif regulon have been shown to be essential for the maturation of nitrogenase [2]. NifS, a cysteine desulphurase, provides elemental sulphur and NifU is the acceptor site for iron and sulphur to build a transient Fe–S cluster, which is subsequently transferred to the nitrogenase [3]. NifU is a modular protein composed of three domains [4]. The N-terminal domain, presenting sequence identity with IscU or ISU proteins from other organisms, is the scaffold for the assembly process. The central domain contains a permanent Fe–S cluster and is supposed to be involved in the transfer of the transient cluster. The C-terminal domain presents a CXXC motif, the role of which in the function of the NifU protein has not been assigned so far. Replacement of the cysteine residues from the CXXC motif by alanine did not affect diazotrophic growth of *Azotobacter vinelandii* [4].

Genetic investigations of Fe–S cluster assembly in yeast allowed important progress in the understanding of this mechanism in eukaryotes [1]. Among the proteins involved in this process in yeast mitochondria, the Nfu1 protein shares significant sequence identity with the C-terminal domain of the NifU protein [5,6]. The 50-amino-acid conserved region containing the CXXC motif can be defined as the NFU domain. *In vitro* comparison of the ability of mitochondrial extracts from wild-type or *Δnfu1* strains in Fe–S cluster assembly revealed a 40 % decrease in the activity of the *Δnfu1* mutant strain [7]. Whereas *Δnfu1* or *Δisu1* strain did not present growth defects, growth of a *Δisu1 Δnfu1* yeast mutant was altered at 34 °C or in the presence of glycerol as a non-fermentable carbon source, indicating a respiratory defect in this strain [5]. Mitochondrial iron accumulation was also detected in *Δisu1 Δnfu1* yeast mitochondria, which appears to be a typical symptom of strains impaired in Fe–S cluster biogenesis. Furthermore, genetic interactions between *NFU1* and *SSQ1* genes were demonstrated in a synthetic lethal screen [5]. Ssq1p is a molecular chaperone dedicated to Fe–S cluster assembly in yeast mitochondria. Altogether, these results are in favour of a role for Nfu1p in Fe–S cluster biogenesis in yeast mitochondria.

In the cyanobacteria *Synechocystis* PCC6803, a single gene (*ssl2667*), named *nifU*, encodes a NFU-like protein presenting sequence identity with the C-terminal domain of *A. vinelandii* NifU protein. This short polypeptide is restricted to the NFU domain containing the CXXC motif, and is capable of accepting and transferring a 2Fe–2S cluster to an apoferredoxin [8]. *ssl2667* is an essential gene for *Synechocystis* PCC6803, suggesting a key role for the corresponding protein in Fe–S cluster assembly in this organism. Nishio and Nakai [8] suggested that *Synechocystis* NFU-like protein could act as a scaffold protein in the assembly mechanism.

In plants, although Fe–S proteins are required for essential processes like photosynthesis or respiration and are present in different cellular compartments, relatively little molecular data are available on Fe–S cluster biogenesis. *Arabidopsis thaliana* genome sequence analysis revealed the existence of several

Abbreviations used: GFP, green fluorescent protein; Ni-NTA, Ni^{2+}-nitrilotriacetate; RRF, ribosome recycling factor; RT, reverse transcriptase; SyNifU, *Synechocystis* NFU protein; YNB, yeast nitrogen base.

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The nucleotide sequence data reported for AtNFU1, AtNFU2, AtNFU3, AtNFU4 and AtNFU5 cDNA sequences will appear in EMBL under the accession numbers AJ512933, AJ512934, AJ512935, AJ512936 and AJ512937 respectively.

genes encoding proteins similar to yeast and bacterial proteins known to be involved in Fe–S biogenesis. In the present study, we have characterized the *A. thaliana* NFU gene family. The Arabidopsis functional NFU proteins are classified into two classes, a mitochondrial form and a plastidial form presenting plant-specific features.

EXPERIMENTAL

Reverse transcriptase (RT)–PCR, cDNA cloning and sequencing

Total RNA was prepared by the guanidine procedure [9] from various tissues of *A. thaliana* plants grown in a greenhouse for 35 days. RT–PCRs were performed as described previously [10]. cDNAs were amplified by PCR with pairs of primers, whose sequences were designed on the basis of the annotated genomic sequences. Sequences of primers used in the present study are available on request. Primers were designed to generate appropriate restriction sites for the cloning of PCR products into the pYPGE15 vector [11]. To target AtNFU1–3 proteins to yeast mitochondria, the sequences encoding residues 1–61 were replaced by the sequence coding for the mitochondrial targeting sequence (residues 1–37) of yeast Atp2p [F(1)F(0)-ATPase complex, β-subunit] [12]. Primers used enabled the cloning of N-terminal Atp2p in pYPGE15 and subsequent cloning of the truncated cDNAs in frame. Each DNA construct has been sequenced (Genome Express, Grenoble, France).

Subcellular localization of AtNFU-green fluorescent protein (GFP) fusion proteins

Primers were designed to amplify the sequences encoding for the AtNFU N-terminal domains (residues 1–111 for AtNFU1, 1–108 for AtNFU2, 1–98 for AtNFU3 and 1–116 for AtNFU4) and allowed their cloning in frame upstream of the GFP-coding region in the pBiGF vector [10] at *Xba*I–*Bam*HI sites. *A. thaliana* protoplasts obtained from cultured cells were transformed using 50 μg of plasmid construct by the polyethylene glycol method as described previously [10].

Transformed cells were incubated at 23 °C for 24 h and were observed by confocal fluorescence microscopy as reported previously [10]. Mitotracker Orange (Molecular Probes, Leiden, The Netherlands) was used at a final concentration of 200 nM and detected using excitation at 568 nm and collection of the signal at 605 nm.

Recombinant NFU2 expression in *Escherichia coli* and raising of antibodies

Primers were designed to amplify part of the coding sequence from AtNFU2 cDNA (nt 171–719) and to allow the cloning in the pET20 vector (Novagen, Madison, WI, U.S.A.) at *Nde*I–*Xho*I sites. BL21 *E. coli* strain was used as a host for overexpression of the recombinant His-tagged protein. Recombinant protein was purified from crude extracts of an overnight culture of transformed *E. coli* grown at 30 °C in Luria–Bertani medium. His-tagged protein was purified using Ni^{2+}-nitrilotriacetate (Ni-NTA) agarose (Qiagen, Valencia, CA, U.S.A.) according to the manufacturer's instructions. Recombinant purified NFU2 protein was loaded on to SDS/PAGE and antibodies were raised in rabbits from gel slices containing the protein (Elevage scientifique des Dombes, Villars les Dombes, France).

Reconstitution of Fe–S cluster

An overnight culture of BL21 *E. coli* cells expressing NFU2-His_6 protein was lysed using B-PER II reagent (Pierce, Rockford, IL, U.S.A.) under anaerobic conditions, and all subsequent steps were also performed in a glove box under argon atmosphere. Cell debris was removed by a 10 min centrifugation at 14000 ***g***. Fe–S cluster was reconstituted 3 h after the addition of 5 mM dithiothreitol, 2 mM cysteine, 2 mM Na_2S and 0.25 mM iron citrate to the supernatant. NFU2 His-tagged protein was purified on Ni-NTA agarose column as mentioned above. UV–visible spectra were performed using Helios spectrophotometer (Thermo Spectronic, Cambridge, U.K.).

Protein-blot analysis

Protein samples were separated by SDS/PAGE and transferred to Hybond-P membrane (Amersham Biosciences, Little Chalfont, U.K.) as described previously [9]. Chemiluminescent detection was performed using Aurora kit (ICN, Costa Mesa, CA, U.S.A.) as recommended by the supplier. Chloroplasts were purified as described by Kunst [13] and mitochondria-enriched fraction was prepared as described in [14].

Yeast culture, strains, mutant construction and transformation

Yeasts were grown in yeast nitrogen base (YNB) medium [2 % (w/v) glucose, 0.17 % YNB and 0.5 % ammonium sulphate] containing the required amino acids. Growth occurred at 30 °C with shaking, unless otherwise indicated.

Yeast haploid strain *Δisu1* (*Matα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*, Kan^R) was purchased from Research Genetics (Huntsville, AL, U.S.A.). The *HIS3* gene was amplified from pRS413 vector [15] with *HIS3*-specific primers bordered by 45-bp sequences complementary to the 5′ and 3′ sequences of *NFU1* gene. A second PCR was run on yeast genomic DNA to amplify two 370-bp flanking sequences upstream and downstream of the *NFU1* gene respectively. These PCR products were mixed and further amplified by PCR to obtain the final construct made of *HIS3* coding sequence bordered by the 370-bp *NFU1* flanking sequences as recombinant ends. Yeast *Δisu1* mutant was transformed with this fragment and selected on a medium depleted of histidine. *NFU1* disruption was checked by PCR. Yeasts were transformed by the lithium acetate method [16].

RESULTS

Cloning of AtNFU cDNAs and primary sequence analysis

To identify *A. thaliana* genes encoding proteins similar to the C-terminal domain of *A. vinelandii* NifU protein, a database search was performed among the predicted proteins in Arabidopsis genome. This database mining enabled us to retrieve five protein sequences presenting significant sequence identity with NifU C-terminal domain, and annotated as NifU-like proteins. The corresponding genes have been named *AtNFU1–5*, and cDNAs have been cloned using an RT–PCR approach. Such a strategy was successfully used for NFU1–4 cDNAs, whereas we were unable to clone NFU5 cDNA. The full-length NFU5 cDNA exists as an expressed sequence tag (N96934) and its clone was provided by Dr P. Nacry (BPMP, Montpellier, France).

Alignment of AtNFU primary sequences with *Saccharomyces cerevisae*, *Rickettsia prowazekii*, *Homo sapiens* and *Synechocystis* PCC6803 NFU proteins reveals a strong conservation of the NFU domain in all of them, and the presence of the CXXC motif

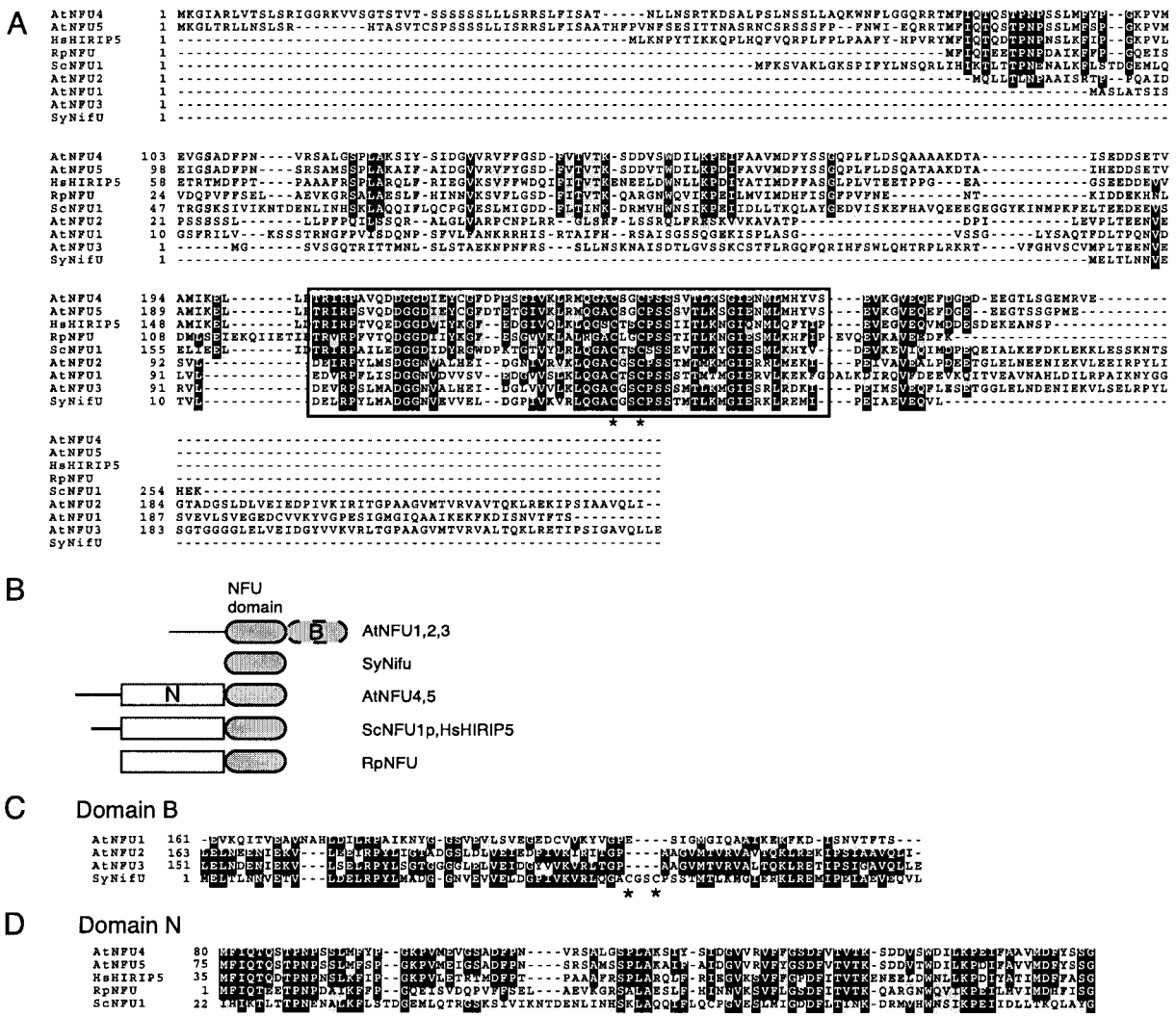

Figure 1 Sequence alignments of NFU proteins

(**A**) AtNFU1–5 protein sequences were aligned along with HIRIP5 (NP_056515) from *H. sapiens* (Hs), NFU (CAA15105.1) from *R. prowazekii* (Rp), Nfu1 (NP_012884) from *S. cerevisiae* (Sc) and NifU (BAA18665) from *Synechocystis* PCC6803 (Sy). The conserved NFU domain is boxed. Stars indicate the cysteine residues from the CXXC motif. (**B**) Schematic representation of NFU proteins aligned in (**A**). (**C**) Comparison of AtNFU1–3 domain B with SyNifU. Stars indicate the cysteine residues from the CXXC motif. (**D**) Alignment of AtNFU4 and AtNFU5, HsHIRIP5, RpNFU and ScNFU1 domain N. Alignments were performed using the PIMA v.1.4 program [30].

(Figure 1A). In contrast, sequences flanking the NFU domain are less conserved and allow us to distinguish two classes of NFU proteins in Arabidopsis.

AtNFU4 and AtNFU5 exhibit more sequence identity when compared with AtNFU1–3 with NFU from yeast, human and α-proteobacterial proteins. In these NFU proteins, the NFU domain is located at the C-terminus of the polypeptide chain, downstream of domain N (Figures 1B and 1D). The latter is specific to this class of NFU proteins, and is absent from AtNFU1–3 primary sequences. Yeast, human and Arabidopsis sequences display an additional sequence at their N-terminus when compared with the *R. prowazekii* NFU protein. Interestingly, ScNFU1 has been shown to be located in yeast mitochondria [5] and *R. prowazekii* has been suggested as the ancestor of mitochondria [17]. Bioinformatic analysis of HsHIRIP5 and AtNFU4 and AtNFU5 protein sequences suggests that the N-terminal regions of these polypeptides are mitochondrial targeting sequences, using MitoProt [18].

NFU1–3 proteins share sequence identity with *Synechocystis* NFU protein (SyNifU), its sequence being restricted to the NFU domain (Figure 1A). However, sequence alignments reveal that Arabidopsis NFU1–3 proteins contain an additional sequence located downstream of the NFU domain. This region, named domain B, shares also a significant sequence identity with SyNifU, suggesting a common ancestor gene for these proteins which has been duplicated to generate plant NFU1–3 genes (Figure 1B). However, domain B is unusual in that the CXXC motif conserved in all NFU domains is absent (Figure 1C). It has to be mentioned that domain B of NFU2 and NFU3 presents 47 % identity with SyNifU, whereas domain B of NFU1 is only 29 % identical with SyNifU indicating a separate evolution. The amino acid sequence located upstream of the NFU domain is also specific to these proteins and is predicted to be a plastid targeting sequence by the ChloroP program [19].

Therefore Arabidopsis NFU proteins can be divided into two classes, NFU4 and NFU5 sharing sequence identity with ScNFU1

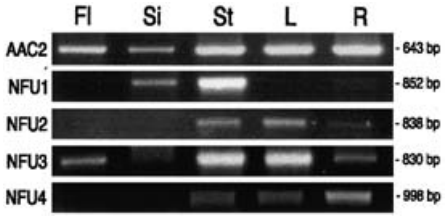

Figure 2 RT–PCR detection of AtNFU1–4 mRNA in various tissues of Arabidopsis

Total RNA was extracted from flowers (Fl), siliques (Si), floral stalk (St), leaves (L) and roots (R) of 35-day-old Arabidopsis plants. Specific primers were used to amplify each cDNA. *Actin-2* mRNA (AAC2) detection was used as a positive control of RNA extraction and R–PCR product sizes are indicated.

mitochondrial protein and other related proteins, whereas NFU1–3 are part of a new class of NFU protein, presenting plant-specific features and predicted to be localized in plastids. Searches in plant DNA databases revealed that this class of NFU proteins is not unique to *A. thaliana*, since similar sequences from other plant species were retrieved, presenting the same characteristics.

Expression pattern of *AtNFU* genes *in planta*

The *AtNFU* gene expression pattern was studied using non-quantitative RT–PCR (Figure 2). RNA was extracted from siliques, flowers, floral stalks, leaves and roots from 35-day-old *A. thaliana* plants. Actin-2 mRNA detection was used as a RT–PCR control. In these plant RNAs, we were not able to detect the NFU5 transcript. However, a NFU5 expressed sequence tag is available in the databases (accession no. N96934), proving that *AtNFU5* gene is indeed expressed *in planta*. NFU4 mRNA signal was observed in roots, leaves and floral stalks. RT–PCR analysis revealed that *NFU1–3* genes are differentially expressed in Arabidopsis tissues. *AtNFU1* gene expression is restricted to floral stalks and siliques in which the NFU1 mRNA is the only NFU transcript detectable. *NFU2* gene expression was observed in floral stalks and leaves, whereas NFU3 mRNA was detected in the same tissues and also in flowers and roots.

Functional complementation of yeast *Δisu1Δnfu1* mutant by AtNFU proteins

Schilke et al. [5] reported the characterization of a *Δisu1Δnfu1 S. cerevisiae* mutant strain presenting a defect in mitochondrial Fe–S cluster assembly. This strain was thermosensitive at 34 °C and did not grow on non-fermentable carbon sources. This growth alteration is linked to an altered respiration as a result of the impaired Fe–S cluster assembly in the yeast mitochondria. A *Δisu1Δnfu1* mutant was constructed in our laboratory by replacing the *NFU1* gene by the *HIS3* gene in a *Δisu1* strain using homologous recombination. Such a strain presented the characteristics of the *Δisu1Δnfu1* mutant kindly provided by Professor Craig (Madison, WI, U.S.A.), specifically a growth defect at 34 °C or in the presence of glycerol as a carbon source (Figures 3A and 4). In contrast, *Δisu1* single mutant was not affected, as described previously [5], opening the possibility to complement functionally the *Δisu1Δnfu1* mutant by expressing AtNFU proteins. AtNFU1–5 cDNAs were cloned into the pYPGE15 vector and expressed in the yeast *Δisu1Δnfu1* mutant. The growth of these strains was investigated at 30 and 34 °C. Suppression of the *Δisu1Δnfu1* phenotype at 34 °C was observed as a result of the expression of AtNFU1, AtNFU4 and

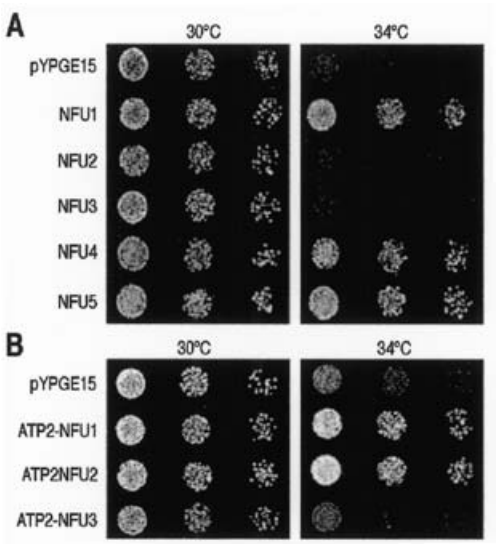

Figure 3 Functional complementation of *Δisu1Δnfu1 S. cerevisiae* mutant deficient in Fe–S cluster assembly by AtNFU proteins

(**A**) *Δisu1Δnfu1* thermosensitive yeast mutant was transformed with an empty vector (pYPGE15) or containing an AtNFU cDNA (NFU1–5). Transformed strains were grown overnight in YNB medium and 5-fold serial dilutions were spotted on YNB-agar mediums (shown from left to right on the Figure). Plates were incubated at the control permissive temperature 30 or 34 °C for 2 days. (**B**) *Δisu1Δnfu1* strain was transformed with Atp2-NFU constructs in which the N-terminal AtNFU putative chloroplast targeting sequence was replaced by the Atp2p yeast mitochondrial targeting sequence and analysed as in (**A**).

AtNFU5 cDNAs (Figure 3A). No growth restoration was detected when AtNFU2 and AtNFU3 were expressed in the mutant strain (Figure 3A). We hypothesized that the putative N-terminal plastid-targeting sequence of these proteins did not allow the AtNFU2 and AtNFU3 proteins to be addressed in yeast mitochondria to complement the *Δisu1Δnfu1* mutant, ScNfu1p being localized in this organelle. To target AtNFU2 and AtNFU3 proteins to yeast mitochondria, the putative N-terminal plastid targeting sequence was replaced by the Atp2p mitochondrial targeting sequence. This Atp2p region has been shown to be necessary and sufficient for efficient targeting of a polypeptide to yeast mitochondria, and the cleavage of the targeting signal [12]. As a control, AtNFU1 N-terminal region was substituted by Atp2p targeting sequence, this protein being, as NFU2 and NFU3, a member of the plant-specific class of NFU proteins. Both AtNFU1 (Figure 3A) and Atp2-NFU1 (Figure 3B) protein expressions were capable of restoring the growth of the *Δisu1Δnfu1* mutant at 34 °C. When targeted to the mitochondria, NFU2 protein was capable of complementing the growth defect of the yeast mutant at 34 °C, whereas no complementation was observed for Atp2-NFU3 (Figure 3B). Using a polyclonal antibody raised against NFU2 protein (see below), we were able to show that NFU3 and Atp2-NFU3 proteins were accumulated at low level and mainly in a truncated form in the yeast strain, when compared with NFU2 and Atp2-NFU2 expressed proteins (results not shown). Such a result could explain the absence of complementation of the *Δisu1Δnfu1* mutant when NFU3 or Atp2-NFU3 proteins were expressed in this strain.

To confirm further this functional complementation assay, growth of the strains expressing AtNFU proteins was investigated on plates using glycerol as a carbon source (Figure 4). Whereas the *Δisu1Δnfu1* mutant did not grow under these conditions due to its respiratory defect, NFU1, NFU4, NFU5 and Atp2-NFU1 and Atp2-NFU2 expressions restored the yeast growth on this

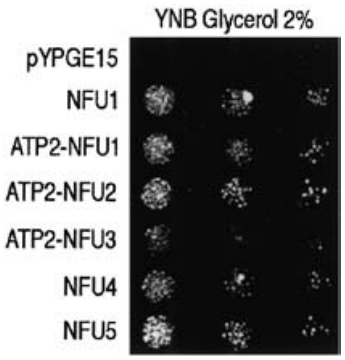

Figure 4 Growth restoration of *Δisu1Δnfu1 S. cerevisiae* mutant on glycerol medium by AtNFU proteins

Δisu1Δnfu1 yeast mutant respiratory defect was used to investigate the restoration of the mutant growth by expression of AtNFU proteins. Transformed strains were grown overnight in YNB medium and 5-fold serial dilutions (shown from left to right on the Figure) were spotted on YNB-agar plates containing 2 % glycerol as non-fermentable carbon source. Strains are as described in Figure 3.

non-fermentable carbon source (Figure 4). Expression of Atp2-NFU3 protein led to a faint growth, when compared with the other strains expressing plant NFU proteins (Figure 4). This result is consistent with the expression of NFU3 protein in a less abundant and truncated form (results not shown). These results are in agreement with the results obtained with similar strains using the thermosensitive phenotype suppression as a marker of functional complementation. Altogether, these results suggest that *A. thaliana NFU1*, *NFU2, NFU4 and NFU5* genes encode functional NFU proteins.

Intracellular localization of AtNFU-GFP fusion proteins

NFU4 and NFU5 are more closely related to the mitochondrial ScNFU1 protein and are predicted to be located in mitochondria, whereas AtNFU1–3 proteins present sequence identity with cyanobacterial SyNifU and are predicted to be targeted to plant plastids. To check this hypothesis, the N-terminal region of these proteins was fused in frame with the GFP and transiently expressed in Arabidopsis cell suspension protoplasts. Confocal microscopy analysis revealed that NFU1–3-GFP fusion proteins are indeed targeted to plastids. The chlorophyll red autofluorescence was used as a chloroplast marker; its pattern matches exactly the one detected for NFU1–3-GFP proteins (Figure 5). These results support the hypothesis that the plant-specific NFU proteins NFU1–3 are located in the plastids. On the contrary, when the N-terminus of NFU4 protein, a member of the other class of Arabidopsis NFU, was expressed fused with the GFP, the green fluorescence pattern detected did not match with the red chlorophyll autofluorescence (Figure 5). The labelling pattern obtained with a mitochondrial-specific dye, Mitotracker Orange, overlapped with the fluorescence pattern displayed by Arabidopsis protoplasts expressing NFU4-GFP fusion protein. The result demonstrates that the NFU4-GFP protein is targeted to the mitochondria in Arabidopsis protoplasts. Therefore Arabidopsis NFU proteins can be separated into two classes that are localized in two distinct intracellular organelles.

Immunodetection of NFU2 and/or NFU3 proteins in chloroplast extracts

Intracellular localization of plant-specific NFU2 and NFU3 subclass of proteins was further investigated using immunodetection. For this purpose, NFU2 protein, expressed by amino acids 58–238, was overexpressed in *E. coli* as an His-tagged protein and polyclonal rabbit antibodies were raised against the purified NFU2 polypeptide. These antibodies were capable of detecting both NFU2 and NFU3 proteins expressed in yeast (results not shown), in agreement with the high sequence identity of these two polypeptides. Total protein from leaves and fractions enriched in chloroplasts and mitochondria were prepared and analysed by protein-blot analysis (Figure 2). Antibodies raised against a spinach chloroplastic ribosome recycling factor (RRF) [20] and the wheat subunit 9 of mitochondrial NADH dehydrogenase [21] were used to determine the purity of the purified organelles. The chloroplastic fraction appeared to be free of mitochondrial contaminations, whereas a low level of chloroplastic proteins was detected in the mitochondria-enriched fraction (Figure 6). Using the antibody raised against NFU2 protein, a signal was detected only in the chloroplast fraction (Figure 6). Such a result confirms the plastidial localization of NFU2 and/or NFU3, two closely related polypeptides of the plant-specific class of NFU proteins.

NFU2 recombinant protein binds a labile 2Fe–2S cluster

To characterize further the subclass of plant-specific NFU proteins, recombinant NFU2 protein was purified under anaerobic conditions after chemical reconstitution of Fe–S clusters. UV–visible spectral analysis of the purified reconstituted protein indicates the presence of a peak at 320 nm and a shoulder at 420 nm (Figure 7), typical of 2Fe–2S clusters [22]. Addition of 10 mM EDTA led to the disappearance of these characteristic peaks, suggesting the presence of a labile Fe–S cluster. This feature has also been reported for SyNifU [8].

DISCUSSION

The NFU domain was first described as the C-terminal domain of NifU proteins from nitrogen-fixing bacteria [4]. This domain of approx. 50 amino acids is also present in a family of proteins named NFU from yeast, human and cyanobacteria that have been shown to be involved in Fe–S cluster biogenesis. In these polypeptides, a strict conservation of the CXXC motif of the NFU domain has been observed.

In the present study, we report the characterization of two classes of Arabidopsis NFU polypeptides. NFU4 and NFU5 are closely related proteins and present a structural organization similar to ScNfu1 mitochondrial protein. The NFU domain is localized at the C-terminus of the protein and is preceded by a conserved domain present in human, yeast and α-proteobacterial NFU proteins (Figure 1). The function of this N domain has not been investigated so far, and the precise function of these NFU proteins is still not clear in yeast mitochondria. However, genetic studies and Fe–S cluster assembly investigations *in vitro* clearly indicate the involvement of ScNfu1 in this process [5,7]. Both NFU4 and NFU5 proteins were capable of complementing a *Δisu1Δnfu1* yeast mutant (Figure 3A). This strain, impaired in Fe–S cluster biogenesis, is thermosensitive at 34 °C and presents a growth alteration on glycerol medium due to respiratory defect [5]. NFU4 and NFU5 are predicted to be located in mitochondria, which was confirmed for NFU4 using GFP fusion analysis (Figure 5). In the last few years, mitochondria have been shown to be the major site for Fe–S biogenesis in yeast for both mitochondrial and cytosolic Fe–S proteins [1]. Arabidopsis genome encodes several other proteins similar to yeast proteins known to be involved in Fe–S cluster biogenesis and predicted to be targeted to

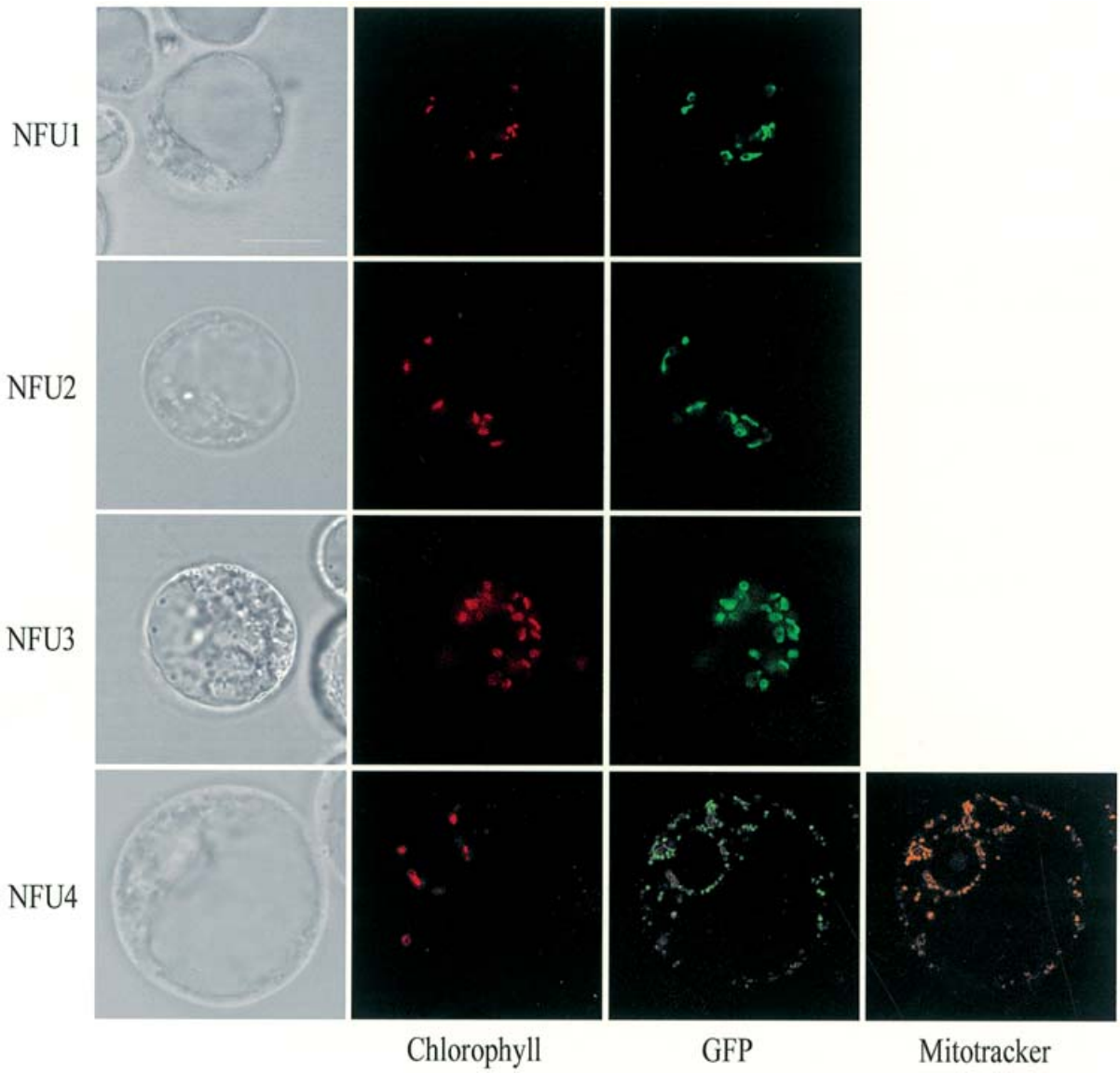

Figure 5 Subcellular localization of AtNFU-GFP fusion proteins

Constructs encoding the AtNFU N-terminal region fused with the GFP were transiently expressed in Arabidopsis cell-suspension culture protoplasts for 24 h. Transformed protoplasts were observed using a confocal laser scanning microscope (MRC1024; Bio-Rad, Hercules, CA, U.S.A.) and a ×60 Plan-Apo oil immersion objective. Light microscopy image of the cell, chlorophyll red autofluorescence used as a chloroplast marker and green fluorescent signals are shown. The cells expressing NFU4-GFP were labelled with Mitotracker Orange mitochondrial marker. Scale bar, 10 μm.

mitochondria. A cysteine desulphurase (AtNFS1) similar to the yeast Nfs1p protein has been shown to be located in plant mitochondria [23] and is a good candidate to transfer the sulphur from cysteine to the nascent Fe–S cluster. The *AtSta1* gene encodes a functional homologue of Atm1p [23]. This ATP-binding-cassette transporter is involved in the export of an unknown substrate required for Fe–S cluster transfer to cytosolic apoproteins in *S. cerevisiae* [24]. These results are in favour of the conservation of a Fe–S cluster assembly mechanism in plant mitochondria as observed in yeast, and a possible role for these organelles to provide Fe–S clusters for plant cytosolic Fe–S proteins.

Plant cells require Fe–S cluster proteins in both mitochondria and plastids for essential processes like respiration and photosynthesis. Indeed, many Fe–S cluster proteins are located in the chloroplasts. Some of them are encoded by the plastid genome, suggesting that Fe–S cluster biogenesis could occur in this compartment. The hypothesis of Fe–S biogenesis in chloroplasts was first proposed by Takahashi et al. [25]. This work showed that chloroplast extracts were capable of converting apo- to holo-ferredoxin using cysteine as a sulphur donor. The characterization of Arabidopsis *NFU* gene family in the present study revealed the existence of a plant-specific class of NFU polypeptides in plastids. AtNFU1–3 are composed of two repeats of the NFU domain, the second having lost the CXXC motif (Figure 1). The N domain of mitochondrial NFU protein is not conserved in these polypeptides, which define a new class of NFU proteins. GFP fusion analysis revealed that they are located in plant plastids (Figure 5). Furthermore, using an antibody raised against NFU2 recombinant protein, a signal was only detected in a leaf

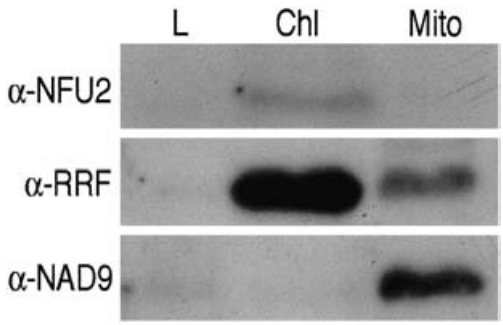

Figure 6 Immunodetection of NFU2 and/or NFU3 proteins in Arabidopsis chloroplast extracts

Protein samples (30 μg) were separated by SDS/PAGE and transferred to Hybond-P membrane. Protein extracts correspond to leaf crude extract (L), chloroplasts (Chl) and mitochondria-enriched fraction (Mito). Chemiluminescent detection was performed using α-NFU2 antibody (1/7500 dilution), α-RRF as chloroplast marker (1/5000) and α-NAD9 as mitochondrial marker (1/50 000). Secondary antibody conjugated to alkaline phosphatase was used at 1/20 000 dilution.

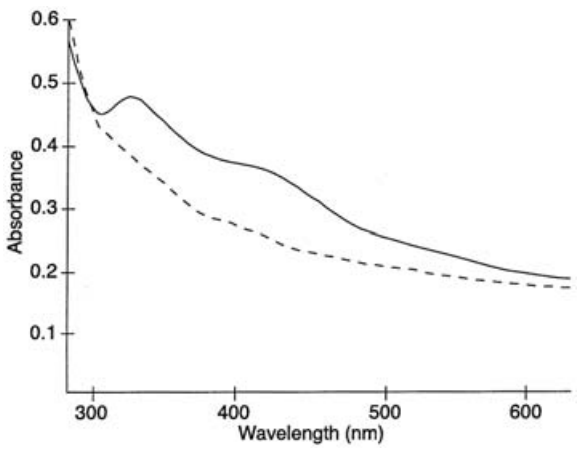

Figure 7 Fe–S cluster reconstitution of NFU2 recombinant protein

Fe–S cluster was reconstituted in bacterial lysate expressing NFU2 recombinant protein under anaerobic conditions. NFU2 was purified on Ni-NTA agarose column and UV–visible spectra were performed on the purified protein before (continuous line) or after 1 h incubation in the presence of 10 mM EDTA (broken line).

chloroplast fraction (Figure 6). All three plastid NFU proteins share a similar organization in primary sequences. However, it should be noted that two subclasses can be distinguished. AtNFU2 and AtNFU3 are closely related, whereas NFU1 is more divergent as shown by the alignment of the B domain of these proteins. In addition, these genes are differentially expressed in plant tissues; for example, NFU1 is the only plastidial NFU expressed in siliques (Figure 2). Such results could reflect a specialized function of these two subclasses or the adaptation to a particular type of plastid. Similar to NFU4 and NFU5, plastid NFU1 and NFU2 proteins fused with a yeast mitochondrial targeting sequence were capable of restoring the growth of the *Δisu1 Δnfu1* yeast mutant at 34 °C or on glycerol medium (Figure 4). NFU1 protein expressed in yeast was capable of restoring the growth of the *Δisu1 Δnfu1* yeast mutant without substitution of its N-terminal targeting sequence by Atp2p N-terminal signal. Study of plant mitochondria import machinery suggests a specific evolution of some of its components to distinguish between mitochondrial and plastid targeting sequences [26]. Yeast mitochondria are less adapted to such discrimination and this could explain that some plastid targeting sequences are capable of addressing proteins in yeast mitochondria. *Δisu1 Δnfu1* strain growth at 34 °C or on glycerol medium was not restored by NFU3 protein, probably due to its accumulation at low level in a truncated form in yeast, although NFU2 and NFU3 are closely related proteins.

The ability of NFU2 protein to bind a labile 2Fe–2S cluster further confirms the link between these new NFU proteins and Fe–S cluster metabolism in plastids. We have recently characterized a NifS-like cysteine desulphurase (AtNFS2) located in plastids, which could be involved in Fe–S biogenesis in this organelle [10]. Another protein related to Fe–S biogenesis, AtISA1 of the IscA protein family, has also been located to Arabidopsis plastids in our laboratory (B. Touraine, S. Léon, J. F. Briat and S. Lobréaux, unpublished work). These proteins as AtNFU1–3 share high sequence identity with the similar cyanobacteria *Synechocystis* PCC6803 proteins. Since cyanobacteria are considered the ancestors of plant plastids [27], such results are in favour of the conservation of a similar Fe–S assembly mechanism in plastids. The unique features of plastid NFU proteins suggest, however, some specificity in the Fe–S biogenesis process. Therefore in plant cells, two different Fe–S assembly mechanisms would exist in two distinct compartments. The structural differences between mitochondrial and plastidial plant NFU proteins suggest that these polypeptides are adapted to distinct Fe–S assembly machineries. Such a divergence could be linked to a major difference in oxygen metabolism in these compartments. Mitochondria are a site for oxygen consumption, whereas chloroplasts produced oxygen through the photosynthetic process. It should be noted that mitochondrial Nfu1p is involved in Fe–S biogenesis, but is not an essential protein for yeast [5]. In contrast, SyNifu, a NFU-like protein whose sequence is restricted to the NFU domain, is essential for *Synechocystis* PCC6803 and has been suggested to be a scaffold for Fe–S biogenesis [8]. This hypothesis is linked to the absence of IscU-like genes in *Synechocystis* genome, which would be the main scaffold protein in *E. coli* and yeast. IscU proteins accept sulphur from NifS-like cysteine desulphurase and are also the binding site for iron to build a Fe–S cluster [28]. The binding of a labile 2Fe–2S cluster by NFU2 protein (Figure 7) would be consistent with such a function in plastids. IscA-like proteins are present in both plant plastids and *Synechocystis* PCC6803 and have been suggested as an alternative scaffold protein to IscU in *E. coli* [29]. The respective functions of plastid IscA-like proteins and AtNFU are yet to be determined. Our results open this perspective and work is in progress in our laboratory to investigate Fe–S biogenesis in plant plastids.

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